Application Note

Amplicon Deep Sequencing on the Illumina MiSeq/NextSeq Platform

Introduction

Amplicon deep-sequencing using next generation sequencing (NGS) technologies has become a powerful tool to study a wide variety of research questions. Typical applications include (i) CRISPR genome editing protocols of eukaryotes, (ii) genome-wide transposon insertion analysis in microorganisms, (iii) Human leukocyte antigen (HLA) typing and (iv) screening of specific somatic mutations in tumor tissues. Common to all these approaches is that a PCR is used to amplify fragments which are then sequenced on a single-molecule level.

The Two-Step PCR Approach - How it Works

The two-step PCR approach combined with Illumina's dual indexing strategy allows to process up to 384 samples in parallel (Figure 1).

The first-step PCR uses primers containing a locus-specific sequence as well as a universal 5' tail as specified in the Nextera library protocol from Illumina (Table 1). Instead of using only one single forward and reverse primer, some protocols make use of up to 3 forward primers that differ in length by adding wobble bases (Ns) between the locus-specific and common 5' tail. This might be especially useful in high-throughput projects where the sequencing throughput is especially critical and many samples are pooled. However, for most projects the sequencing throughput is high enough by simply using one forward and one reverse primer. If you need further background about this particular topic please contact us.

The resulting PCR amplicons are then used as templates within the second-step PCR for further amplification but also to include the indexes (barcodes) as well as the Illumina adaptors. The Illumina indexing strategy for the second-step PCR consists of 16 forward primers and 24 reverse primers. The combinatorial use of these primers (16 x 24) defines the maximal number of 384 samples which can be pooled and sequenced on one Illumina MiSeq/NextSeq run.

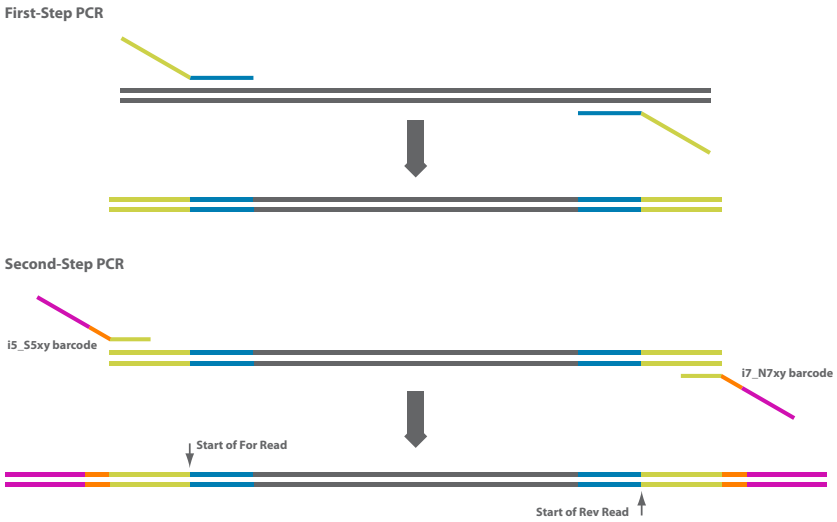

Figure 1. Overview of the double indexing strategy used in the Illumina two-step protocol. During the Illumina sequencing step the amplified genomic sequence including the specific primers (grey and blue bars) as well as the forward and reverse barcodes (orange bar) are read-out.

Table 1. 5' tails used for the first-step PCR. Oligonucleotide sequences

PrimerName	Sequence (5'-3')
1st_PCR_for	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus-specific sequence]
1st_PCR_rev	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locus-specific sequence]

Table 2. Indexed forward primers for the second-step PCR. Oligonucleotide sequences © 2007-2013 Illumina, Inc. All rights reserved.

PrimerName	Sequence (5'-3')	Index Name	Index Sequence
NGS_i5_S502	AATGATACGGCGACCACCGAGATCTACAC**CTCTCTAT**TCGTCGGCAGCGTC	S502	CTCTCTAT
NGS_i5_S503	AATGATACGGCGACCACCGAGATCTACAC**TATCCTCT**TCGTCGGCAGCGTC	S503	TATCCTCT
NGS_i5_S505	AATGATACGGCGACCACCGAGATCTACAC**GTAAGGAG**TCGTCGGCAGCGTC	S505	GTAAGGAG
NGS_i5_S506	AATGATACGGCGACCACCGAGATCTACAC**ACTGCATA**TCGTCGGCAGCGTC	S506	ACTGCATA
NGS_i5_S507	AATGATACGGCGACCACCGAGATCTACAC**AAGGAGTA**TCGTCGGCAGCGTC	S507	AAGGAGTA
NGS_i5_S508	AATGATACGGCGACCACCGAGATCTACAC**CTAAGCCT**TCGTCGGCAGCGTC	S508	CTAAGCCT
NGS_i5_S510	AATGATACGGCGACCACCGAGATCTACAC**CGTCTAAT**TCGTCGGCAGCGTC	S510	CGTCTAAT
NGS_i5_S511	AATGATACGGCGACCACCGAGATCTACAC**TCTCTCCG**TCGTCGGCAGCGTC	S511	TCTCTCCG
NGS_i5_S513	AATGATACGGCGACCACCGAGATCTACAC**TCGACTAG**TCGTCGGCAGCGTC	S513	TCGACTAG
NGS_i5_S515	AATGATACGGCGACCACCGAGATCTACAC**TTCTAGCT**TCGTCGGCAGCGTC	S515	TTCTAGCT
NGS_i5_S516	AATGATACGGCGACCACCGAGATCTACAC**CCTAGAGT**TCGTCGGCAGCGTC	S516	CCTAGAGT
NGS_i5_S517	AATGATACGGCGACCACCGAGATCTACAC**GCGTAAGA**TCGTCGGCAGCGTC	S517	GCGTAAGA
NGS_i5_S518	AATGATACGGCGACCACCGAGATCTACAC**CTATTAAG**TCGTCGGCAGCGTC	S518	CTATTAAG
NGS_i5_S520	AATGATACGGCGACCACCGAGATCTACAC**AAGGCTAT**TCGTCGGCAGCGTC	S520	AAGGCTAT
NGS_i5_S521	AATGATACGGCGACCACCGAGATCTACAC**GAGCCTTA**TCGTCGGCAGCGTC	S521	GAGCCTTA
NGS_i5_S522	AATGATACGGCGACCACCGAGATCTACAC**TTATGCGA**TCGTCGGCAGCGTC	S522	TTATGCGA

Table 3. Indexed reverse primers for the second-step PCR. Oligonucleotide sequences © 2007-2013 Illumina, Inc. All rights reserved.

PrimerName	Sequence (5'-3')	Index Name	Index Sequence
NGS_i7_N701	CAAGCAGAAGACGGCATACGAGAT**TCGCCTTA**GTCTCGTGGGCTCGG	N701	TCGCCTTA
NGS_i7_N702	CAAGCAGAAGACGGCATACGAGAT**CTAGTACG**GTCTCGTGGGCTCGG	N702	CTAGTACG
NGS_i7_N703	CAAGCAGAAGACGGCATACGAGAT**TTCTGCCT**GTCTCGTGGGCTCGG	N703	TTCTGCCT
NGS_i7_N704	CAAGCAGAAGACGGCATACGAGAT**GCTCAGGA**GTCTCGTGGGCTCGG	N704	GCTCAGGA
NGS_i7_N705	CAAGCAGAAGACGGCATACGAGAT**AGGAGTCC**GTCTCGTGGGCTCGG	N705	AGGAGTCC
NGS_i7_N706	CAAGCAGAAGACGGCATACGAGAT**CATGCCTA**GTCTCGTGGGCTCGG	N706	CATGCCTA
NGS_i7_N707	CAAGCAGAAGACGGCATACGAGAT**GTAGAGAG**GTCTCGTGGGCTCGG	N707	GTAGAGAG
NGS_i7_N710	CAAGCAGAAGACGGCATACGAGAT**CAGCCTCG**GTCTCGTGGGCTCGG	N710	CAGCCTCG
NGS_i7_N711	CAAGCAGAAGACGGCATACGAGAT**TGCCTCTT**GTCTCGTGGGCTCGG	N711	TGCCTCTT
NGS_i7_N712	CAAGCAGAAGACGGCATACGAGAT**TCCTCTAC**GTCTCGTGGGCTCGG	N712	TCCTCTAC
NGS_i7_N714	CAAGCAGAAGACGGCATACGAGAT**TCATGAGC**GTCTCGTGGGCTCGG	N714	TCATGAGC
NGS_i7_N715	CAAGCAGAAGACGGCATACGAGAT**CCTGAGAT**GTCTCGTGGGCTCGG	N715	CCTGAGAT
NGS_i7_N716	CAAGCAGAAGACGGCATACGAGAT**TAGCGAGT**GTCTCGTGGGCTCGG	N716	TAGCGAGT
NGS_i7_N718	CAAGCAGAAGACGGCATACGAGAT**GTAGCTCC**GTCTCGTGGGCTCGG	N718	GTAGCTCC
NGS_i7_N719	CAAGCAGAAGACGGCATACGAGAT**TACTACGC**GTCTCGTGGGCTCGG	N719	TACTACGC
NGS_i7_N720	CAAGCAGAAGACGGCATACGAGAT**AGGCTCCG**GTCTCGTGGGCTCGG	N720	AGGCTCCG
NGS_i7_N721	CAAGCAGAAGACGGCATACGAGAT**GCAGCGTA**GTCTCGTGGGCTCGG	N721	GCAGCGTA
NGS_i7_N722	CAAGCAGAAGACGGCATACGAGAT**CTGCGCAT**GTCTCGTGGGCTCGG	N722	CTGCGCAT
NGS_i7_N723	CAAGCAGAAGACGGCATACGAGAT**GAGCGCTA**GTCTCGTGGGCTCGG	N723	GAGCGCTA
NGS_i7_N724	CAAGCAGAAGACGGCATACGAGAT**CGCTCAGT**GTCTCGTGGGCTCGG	N724	CGCTCAGT
NGS_i7_N726	CAAGCAGAAGACGGCATACGAGAT**GTCTTAGG**GTCTCGTGGGCTCGG	N726	GTCTTAGG
NGS_i7_N727	CAAGCAGAAGACGGCATACGAGAT**ACTGATCG**GTCTCGTGGGCTCGG	N727	ACTGATCG
NGS_i7_N728	CAAGCAGAAGACGGCATACGAGAT**TAGCTGCA**GTCTCGTGGGCTCGG	N728	TAGCTGCA
NGS_i7_N729	CAAGCAGAAGACGGCATACGAGAT**GACGTCGA**GTCTCGTGGGCTCGG	N729	GACGTCGA

How to Order the NGS Primers?

Download first the specific "OrderForm_IlluminaAmpliconDeepSeq" from Microsynth's website (see Amplicon Deep Sequencing within the NGS menu). Specify now the locus-specific sequences for your first-step PCR primers and then select your desired indexed forward and reverse primers. Send the upload sheet to info@microsynth.ch and request your specific offer. Alternatively, directly order the oligos in our webshop using the prefix "NGS_" in the 0.2 µmol scale, HPLC purified.

Example of Primer Pipetting Scheme for 96 Samples (8 x 12 indexes)

Table 4. Pipetting scheme for barcoding 96 samples using 8 i5 indexes (vertical) and 12 i7 indexes (horizontal). Using the 16 x 24 described indexes of Illumina it is also possible to unequivocally indentify up to 384 samples.

	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
S502	S502	S502	S502	S502	S502	S502	S502	S502	S502	S502	S502	S502
	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
S503	S503	S503	S503	S503	S503	S503	S503	S503	S503	S503	S503	S503
	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
S505	S505	S505	S505	S505	S505	S505	S505	S505	S505	S505	S505	S505
	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
S506	S506	S506	S506	S506	S506	S506	S506	S506	S506	S506	S506	S506
	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
S507	S507	S507	S507	S507	S507	S507	S507	S507	S507	S507	S507	S507
	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
S508	S508	S508	S508	S508	S508	S508	S508	S508	S508	S508	S508	S508
	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
S510	S510	S510	S510	S510	S510	S510	S510	S510	S510	S510	S510	S510
	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
S511	S511	S511	S511	S511	S511	S511	S511	S511	S511	S511	S511	S511
	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715

PCR Design Considerations

In general, the first-step PCR is a standard PCR using a proofreading polymerase and 5' tailed PCR primers. The only point to consider is the length of the amplified product including the locus-specific parts of the forward and reverse primers. If the sequence of the entire amplicon is of interest, the Illumina forward and reverse reads are stitched. This requires for overlap ≥50 bases of the forward and reverse reads (Table 5).

Illumina Read Length	Max. Fragment Length	Illumina Platform
2 * 150	250	MiSeq/NextSeq
2 * 250	450	MiSeq
2 * 300	550	MiSeq

Table 5. Various Illumina read lengths specifications and their corresponding maximal amplicon sequencing lengths. Single-end run configurations are also possible depending on your specific project. Please contact us to discuss these possibilities.

Microsynth Competences and Services

One of Microsynth's core skills is in the field of amplicon deep sequencing. Microsynth is able to offer its customers a non-stop service covering the entire process from experimental design planning, DNA isolation, PCR amplification and sequencing up to bioinformatics analysis of the generated data for typical deep sequencing projects (Figure 2).

DNA Isolation: Either the customer provides isolated DNA or outsources this step to Microsynth (>13 years of experience in DNA/RNA isolation from various and demanding matrices).

PCR Amplification: The PCR amplification will use a state-of the-art high-fidelity polymerase resulting in high-quality multiplex amplicon libraries. The customer either provides the DNA, the first-step PCR products, or the second-step products. The advantage of providing the first-step PCR products is that you will only need two primers per locus and do not need to order any indexed primers.
PCR products are purified, quantified with fluorescence spectroscopy using Picogreen and pooled in equimolar amounts.

NGS Sequencing: Sequencing is done using Illumina MiSeq/nextSeq sequencing technology. Both technologies allow high-throughput profiling at low costs and the MiSeq has the additional advantage of long reads (up to 550 bp).

Bioinformatics Analyses: Depending on customer requirements Microsynth can offer customized analysis of the data including QC of the sequence data, stitching, demultiplexing, mapping and/or identification of indels and SNPs.

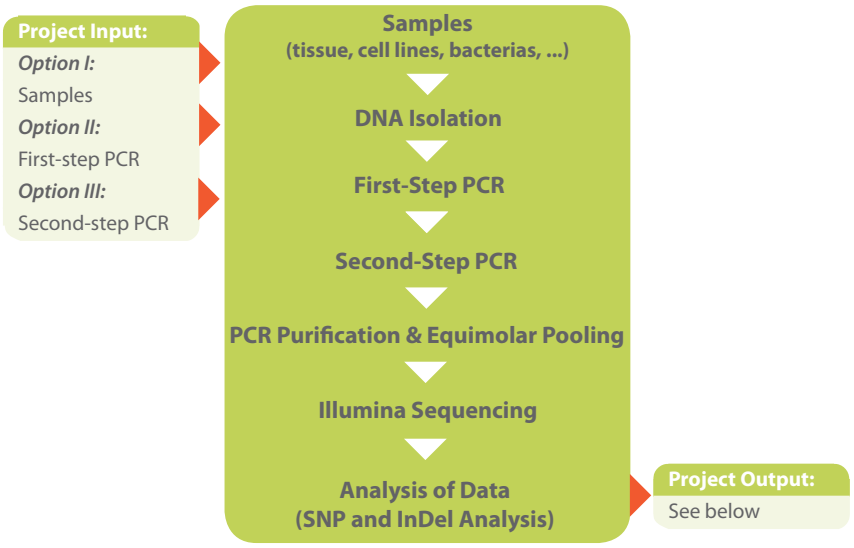

Figure 2. Typical steps in a amplicon deep-sequencing project. Depending on researcher needs Microsynth can deliver a non-stop service starting from DNA isolation, over PCR amplification and sequencing to customized data analysis. Further, customer either sends samples for DNA isolation, first-step PCR or second-step PCR products or pooled ready-to-sequence libraries.

Example Outputs for an Amplicon Deep-Sequencing Project

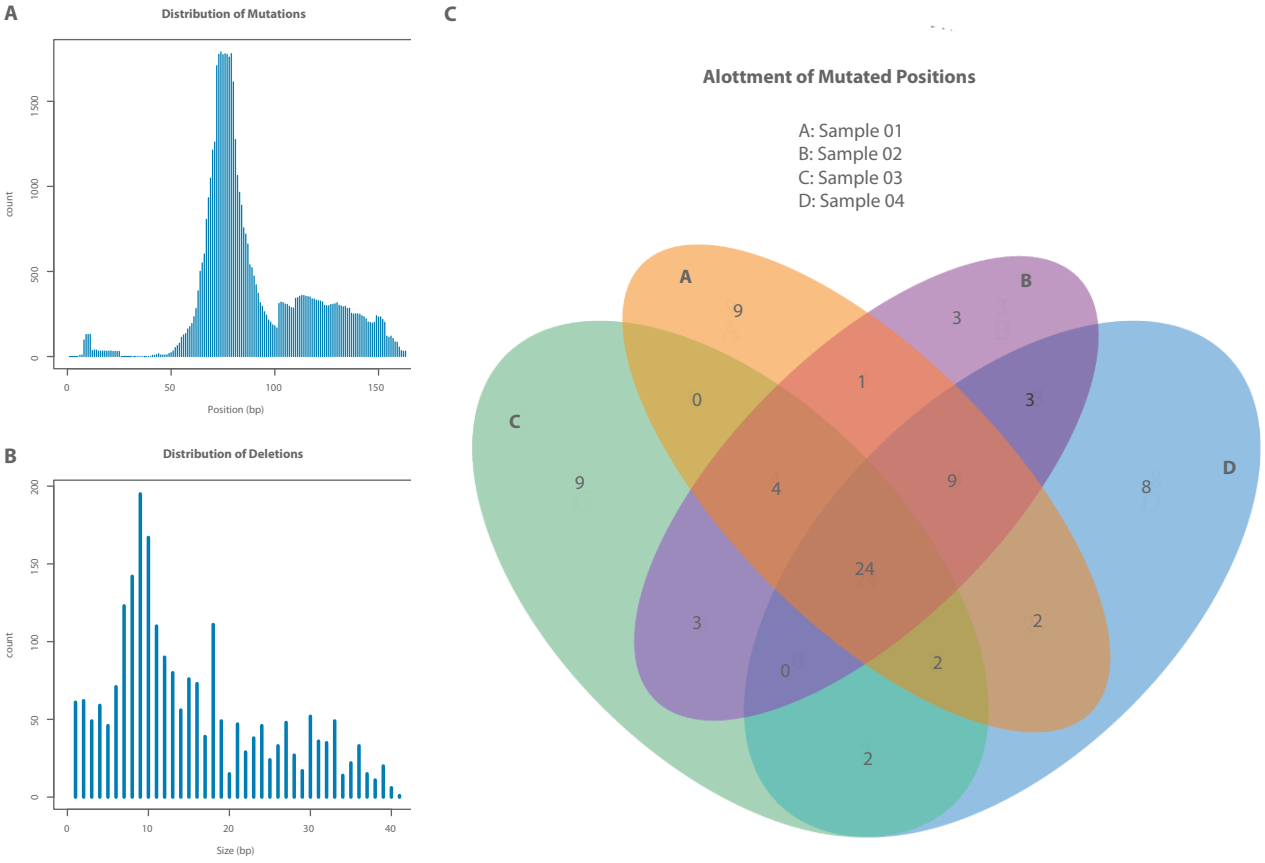

Figure 3. Example output of a deep-sequencing project. Sequencing reads of individual samples are aligned to the reference and the distribution of SNPs (**3A**) and Indels (**3B**) are reported. In addition, Veen diagrams are displayed to visualize the number of mutations shared by the different samples included in the analysis (**3C**).